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Research Paper

Increased mitochondrial superoxide in the brain, but not periphery, sensitizes mice to angiotensin II-mediated hypertension

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ABSTRACT

Angiotensin II (AngII) elicits the production of superoxide ($O_2^{\cdot-}$) from mitochondria in numerous cell types within peripheral organs and in the brain suggesting a role for mitochondrial-produced $O_2^{\cdot-}$ in the pathogenesis of hypertension. However, it remains unclear if mitochondrial $O_2^{\cdot-}$ is causal in the development of AngII-induced hypertension, or if mitochondrial $O_2^{\cdot-}$ in the absence of elevated AngII is sufficient to increase blood pressure. Further, the tissue specific (*i.e.* central versus peripheral) redox regulation of AngII hypertension remains elusive. Herein, we hypothesized that increased mitochondrial $O_2^{\cdot-}$ in the absence of pro-hypertensive stimuli, such as AngII, elevates baseline systemic mean arterial pressure (MAP), and that AngII-mediated hypertension is exacerbated in animals with increased mitochondrial $O_2^{\cdot-}$ levels. To address this hypothesis, we generated novel inducible knock-down mouse models of manganese superoxide dismutase (MnSOD), the $O_2^{\cdot-}$ scavenging antioxidant enzyme specifically localized to mitochondria, targeted to either the brain subfornical organ (SFO) or peripheral tissues. Contrary to our hypothesis, knock-down of MnSOD either in the SFO or in peripheral tissues was not sufficient to alter baseline systemic MAP. Interestingly, when mice were challenged with chronic, peripheral infusion of AngII, only the MnSOD knock-down confined to the SFO, and not the periphery, demonstrated an increased sensitization and potentiated hypertension. In complementary experiments, over-expressing MnSOD in the SFO significantly decreased blood pressure in response to chronic AngII. Overall, these studies indicate that mitochondrial $O_2^{\cdot-}$ in the brain SFO works in concert with other AngII-dependent factors to drive an increase in MAP, as elevated mitochondrial $O_2^{\cdot-}$ alone, either in the SFO or peripheral tissues, failed to raise baseline blood pressure.

1. Introduction

Reactive oxygen species (ROS) such as superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and peroxynitrite ($ONOO^{\cdot-}$) have been implicated in the pathogenesis of angiotensin II (AngII)-mediated hypertension [1,2]. While the downstream reactions of these ROS are distinctly unique, the derivation of these ROS primarily begins with a one electron transfer to oxygen forming $O_2^{\cdot-}$, followed by the dismutation to H_2O_2 or the rapid reaction with nitric oxide (NO^{\cdot}) to form $ONOO^{\cdot-}$ [3]. Thus, the fundamental understanding of how $O_2^{\cdot-}$ is involved in AngII-mediated hypertension is at the crux of deciphering the redox-regulation of this disease.

Superoxide may be produced from various subcellular sources in response to AngII, but mitochondria have been demonstrated to be a primary source in various cell types [2,4–9]. For example, we have demonstrated that AngII increases mitochondrial localized $O_2^{\cdot-}$ in

cultured neurons [7,9]. This increase in mitochondrial $O_2^{\cdot-}$ contributes to the AngII-induced inhibition of outward K^+ current as over-expressing manganese superoxide dismutase (MnSOD), the mitochondrial-targeted $O_2^{\cdot-}$ scavenging antioxidant enzyme, attenuates this AngII-induced neuronal response [7]. Additionally, it has been demonstrated that over-expression of MnSOD in the subfornical organ (SFO), a well-established AngII-sensitive cardiovascular control region in the brain [10,11], in mice attenuates the acute pressor response to centrally administered AngII [10]. Likewise, whole body over-expression of MnSOD or systemic infusion of the mitochondrial-targeted antioxidant MitoTempol has also been shown to attenuate chronic AngII-mediated hypertension in mice [8]. Conversely, heterozygous MnSOD mice become more hypertensive compared to wild-type when aged or challenged with high salt [12]. Overall, these previous studies are highly suggestive that mitochondrial $O_2^{\cdot-}$ contributes to the pathogenesis of hypertension; however, it remains unclear if increased

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mitochondrial $O_2^{\cdot-}$ in the absence of pro-hypertensive stimuli is sufficient to increase systemic blood pressure. Further, because whole body over-expression models of MnSOD or systemic MnSOD heterozygote mice have been utilized, it remains uncertain which cell types or organs are responsible in mediating changes in blood pressure.

Due to these gaps in knowledge, we set out to examine the tissue-specific effects of increased mitochondrial $O_2^{\cdot-}$ on basal and AngII-challenged hemodynamics. With observations from our lab and others suggesting mitochondrial $O_2^{\cdot-}$ is a critical signaling molecule in virtually all cell types, we hypothesized that both central and peripheral increases in mitochondrial $O_2^{\cdot-}$ elevate baseline systemic MAP, and moreover sensitize mice to AngII-mediated hypertension. To test this hypothesis, we utilized a homozygous conditional MnSOD knock-down mouse (loxP derived; MnSOD^{L/L}) combined with two strategies for targeting cre-recombinase to generate peripheral or brain SFO MnSOD knock-down animals. While we observed significant knock-down of MnSOD in both models, contrary to our hypothesis, neither model demonstrated any change in MAP at baseline. However, when challenged with AngII only the brain SFO MnSOD knock-down animals displayed a potentiated hypertensive response, thus suggesting mitochondrial $O_2^{\cdot-}$ in the SFO plays a preferential role over the periphery in sensitizing to AngII-mediated hypertension.

2. Materials and methods

2.1. Mice

All experiments were performed using 8–12 week-old male mice of a C57BL/6 background. Wild-type mice (*i.e.* C57BL/6NHsd) were purchased from Harlan Laboratories/Envigo (Indianapolis, IN). Mice possessing loxP elements flanking (*i.e.* floxed) exon 3 of the MnSOD gene locus (*i.e.* B6.Cg-Sod2^{tm1Lox}; shorthand MnSOD^{L/L}) have been previously described [13]. Mice possessing a conditionally-expressed tamoxifen-inducible cre-recombinase targeted to the ubiquitously expressed ROSA26 gene locus (*i.e.* B6.129-Gt(ROSA)26Sor^{tm1(cre/ERT2)Tyj/J}; shorthand ROSA-Cre^{+/+}) have been previously described and were purchased from Jackson Laboratories (Bar Harbor, ME) [14]. MnSOD^{L/L} and ROSA-Cre^{+/+} mice were backcrossed to the F3 generation to allow for 100% usable progeny of either MnSOD^{L/L} ROSA-Cre^{+/+} (inducible knock-down) or MnSOD^{L/L} ROSA-Cre^{-/-} (control) genotypes. Mice were given access to standard chow (Teklad Laboratory Diet #7012, Harlan Laboratories, Madison, WI) and water *ad libitum*. For all survival surgical procedures, mice were anesthetized using 0.5–2% isoflurane supplemented with 1 l/min oxygen. Bupivacaine (0.5% solution) was used as post-surgical anesthetic, and mice were monitored daily post-operation for signs of illness or infection. Mice were euthanized by pentobarbital overdose (150 mg/kg, Fatal Plus, Vortech Pharmaceuticals, Dearborn, MI) administered intraperitoneally. All procedures were reviewed and approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee.

2.2. Knock-down and over-expression of MnSOD

Protocol 1: For peripheral knock-down of MnSOD, MnSOD^{L/L} ROSA-Cre^{+/+} and ^{-/-} mice were treated daily with tamoxifen (Sigma-Aldrich #T5648, St. Louis, MO) for 5 days. Tamoxifen was resuspended at 20 mg/mL in sunflower seed oil (Sigma-Aldrich #S5007, St. Louis, MO) and injected at 150 mg/kg intraperitoneally per mouse per day. While the ROSA26 promoter is expressed in all cell types, negligible central nervous recombination has been observed in this model after tamoxifen administration [15], thus allowing for an efficient peripheral knock-down of MnSOD. Protocol 2: For knock-down of MnSOD in the brain SFO, MnSOD^{L/L} mice were administered adenovirus (4×10⁷ plaque-forming units (PFU)) encoding either cre-recombinase (AdCre; The University of Iowa Viral Vector Core Facility, Iowa City, IA) or control adenovirus (AdGFP or AdEmpty, collectively AdControl; The

University of Iowa Viral Vector Core Facility, Iowa City, IA) by a one-time intracerebroventricular (ICV) injection. Stereotactic coordinates, as previously described [16], were used for ICV injections. Protocol 3: For over-expression of MnSOD in the SFO, wild-type mice were administered adenovirus (4×10⁷ PFU) encoding human MnSOD (AdMnSOD, ViraQuest Inc., North Liberty, IA) or AdControl by a one-time ICV injection.

2.3. Mean arterial pressure recording

Induction of hypertension was performed by implantation of subcutaneous osmotic minipumps (Alzet #1002, Durect Corporation, Cupertino, CA) delivering AngII (400 ng/kg/min; Sigma #A9525, St. Louis, MO) until complete emptying of the pumps (approximately 3 weeks) [17]. Telemetric recording of mouse hemodynamics has been previously described in detail [17]. Briefly, blood pressure recordings were performed using intra-carotid arterial catheters (PA-C10, Data Sciences International, Minneapolis, MN) attached to radio telemeters for direct measurement of mean arterial pressure and heart rate in conscious unrestrained animals. Hemodynamic recordings were performed for 20 s every minute for the same 2-h time period daily for the duration of the experiment. Averages of mean arterial pressure were calculated daily over the 2-h period when the mice displayed minimal activity.

2.4. Tissue isolation

Analysis of tissues for knock-down studies occurred 14 days after gene recombination (*i.e.* 14 days after the last tamoxifen injection for peripheral tissues or virus administration for SFO) to allow endogenous MnSOD protein to degrade, while tissue analysis in over-expression studies was undertaken 4 days after AdMnSOD injection to allow production of exogenous MnSOD protein. At time of euthanasia, blood was removed from the tissues by 0.9% saline solution perfused via peristaltic pump through the left ventricle of the heart. Following saline perfusion, tissues were either isolated for use in assays requiring live cells or further perfused using a 4% paraformaldehyde solution to fix tissues prior to isolation and analysis.

2.5. Laser-capture microdissection and quantitative reverse transcription real-time PCR (qRT-PCR)

Freshly isolated and non-fixed brains were snap frozen using dry ice and mounted in blocks using OCT freeze media (Thermo Fisher Scientific #23-730-571, Waltham, MA). Brains were cut by cryostat to the level of the SFO and 50 µm sections were placed upon RNase-free frame-foiled polyethylene terephthalate slides (North Central Instruments #11505190, Plymouth, MN). The SFO region was imaged, mapped, and dissected on a Leica LMD7000 laser microdissection microscope. Collected tissue was immediately placed into TRIzol Reagent (Thermo Fisher Scientific #15596-018, Waltham, MA), and total RNA was extracted via manufacturer's instructions. Concentration of RNA was determined spectrophotometrically using a Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA), and cDNA was obtained using the high capacity cDNA archive kit (Applied Biosystems #4368813, Grand Island, NY). SYBR green qRT-PCR for MnSOD was performed on a BioRad iCycler iQ Real-Time PCR Detection System (BioRad, Hercules, California). A threshold in the linear range of PCR amplification was selected and the cycle threshold (Ct) determined. Levels of transcripts were then normalized to the 18S loading control and compared relative to the control sample using the 2^{-ΔΔCt} method. Primers specific to the coding region of either mouse or human MnSOD were used. Primer sequences were as follows: 18S forward, 5'-GCCGAAGCGTTTACTTTGA-3'; 18S reverse, 5'-TCATGGCCTCAGTTCGAA-3'; mouse MnSOD forward, 5'-GCTCTGCCAAGGGAGATGT-3'; mouse MnSOD reverse, 5'-

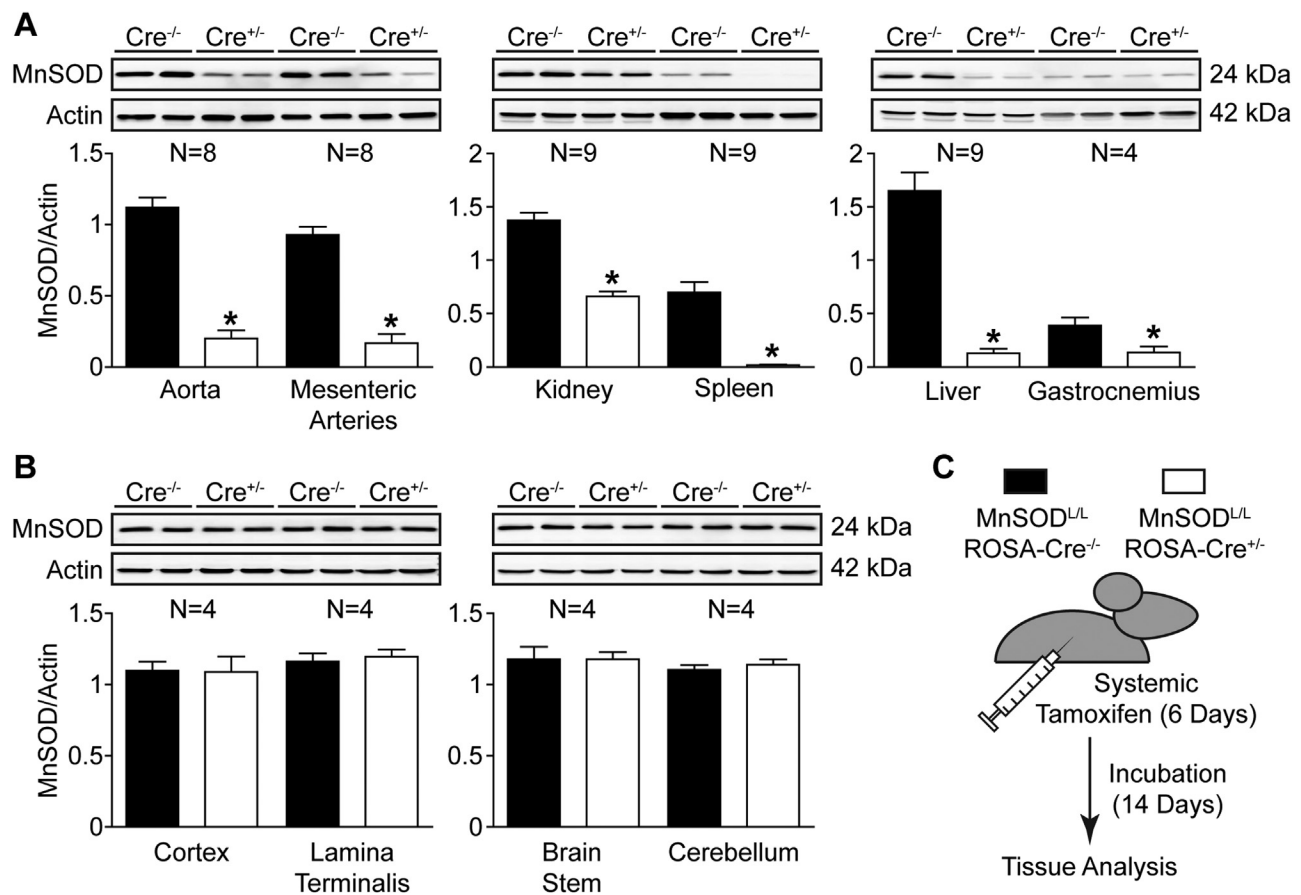


Fig. 1. MnSOD is only knocked-down in peripheral tissues of the ROSA-Cre tamoxifen inducible mouse model. Mice were treated with tamoxifen (150 mg/kg) daily for 6 days followed by a 14-d incubation period to allow endogenous MnSOD to turnover prior to tissue harvest. **A.** Representative western blots and quantification of MnSOD protein in various peripheral tissues. **B.** Representative western blots and quantification of MnSOD protein in regions of the brain. **C.** Schematic of experimental setup and timeline. * $p < 0.05$ vs. MnSOD^{L/L} ROSA-Cre^{-/-}.

GGGCTCAGGTTTGTCCAGAAA-3'; human MnSOD forward, 5'-GGCCTACGTGAACAACCTGAA-3'; human MnSOD reverse, 5'-CTGTAACATCTCCCTTGCCA-3'.

Fluorescent images were acquired with a Zeiss 510 Meta Confocal Laser Scanning Microscope using settings appropriate for GFP and Alexa Fluor 594.

2.6. Western blotting

Immunoblotting was carried out as previously described [18]. Briefly, freshly perfused non-fixed tissues were sonicated in buffered solution containing protease inhibitors (Sigma #P8340, St. Louis, MO). After separation by polyacrylamide gel electrophoresis and transfer to nitrocellulose, membranes were incubated with a 1:1000 dilution one of the following primary antibodies: CuZnSOD (Santa Cruz Biotechnology, CA); MnSOD (Upstate Biotech/Millipore, Billerica, MA) or β -Actin (Sigma, St. Louis, MO). After appropriate secondary antibody staining and washout, images of membranes were acquired by a UVP Bioimaging System (UVP LLC, Upland, CA). All three primary antibodies were utilized on the same membrane by stripping (Thermo Fisher Scientific #21059, Waltham, MA) and re-probing to control for appropriate loading of samples (see [Supplementary Fig. 1](#) for representative uncropped image of complete western blot).

2.7. Immunofluorescence

Fixed brain sections were subjected to standard immunofluorescence as previously described [9]. Briefly, brains were sectioned by cryostat at 20 μ m to expose the SFO. Sections were permeabilized and incubated with MnSOD primary antibody (1:200 dilution, Upstate Biotech/Millipore, Billerica, MA) followed by an Alexa Fluor 594 secondary antibody (1:200, anti-rabbit, Invitrogen, Carlsbad, CA).

2.8. Superoxide analysis

Freshly isolated, frozen, and non-fixed brains were sectioned by cryostat at 20 μ m to expose the SFO. Sections were incubated with 10 μ M fresh non-oxidized dihydroethidium (DHE; VWR #101447-534, Chicago, IL) or MitoSOX Red (Thermo Fisher Scientific #M36008, Waltham, MA) for 30 min at 37 °C. Solution containing DHE or MitoSOX was removed, and slides were imaged on a Zeiss 510 Meta Confocal Laser Scanning Microscope (excitation 488 nm, emission 600 nm). Fluorescence intensity was quantified utilizing ZEN 2010 B SP1 and Image J software.

2.9. Statistics

Data are presented as mean \pm standard error of the mean (SEM). Assessments of blood pressure were performed using 2-way ANOVA followed by Bonferroni correction post hoc test. For two group comparisons, Student's *t*-test was used. GraphPad Prism 5.0 statistical and graphing software was used for all analyses. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Peripheral knock-down of MnSOD does not affect blood pressure response to AngII

To generate a model of peripherally increased mitochondrial $O_2^{\bullet-}$, we bred a conditional MnSOD knock-down mouse (MnSOD^{L/L}) to a mouse expressing a tamoxifen-inducible cre-recombinase under the ubiquitous ROSA26 promoter (ROSA-Cre^{+/-}). While the ROSA26 promoter is expressed in all cell types, minimal central nervous system recombination has been observed in this model after tamoxifen administration [15], thus allowing for an efficient peripheral knock-down of MnSOD. This methodology to create a temporally and spatially-controlled knock-down was utilized due to the embryonic knock-out of MnSOD being lethal shortly after birth, and thus not allowing for adult animals to be studied [19,20].

We first set out to characterize the efficiency of MnSOD knock-down in our conditional peripheral MnSOD knock-down mice (MnSOD^{L/L} ROSA-Cre^{+/-}). After tamoxifen administration (Fig. 1C), tissues were harvested for MnSOD protein analysis. Peripheral tissues demonstrated robust and significant knock-down of MnSOD (Fig. 1A); however, none of the brain regions examined showed any appreciable change in MnSOD protein levels (Fig. 1B). These data confirm the observation that the MnSOD^{L/L} ROSA-Cre^{+/-} mice accurately reflects a mouse model of peripheral MnSOD knock-down, as opposed to a complete whole-body knock-down. Furthermore, no change in the cytoplasmic form of SOD (i.e. copper/zinc SOD, CuZnSOD) was observed in the MnSOD^{L/L} ROSA-Cre^{+/-} mice suggesting the knock-down of MnSOD in peripheral tissues is specific and not counteracted by a compensatory $O_2^{\bullet-}$ removal mechanism (Fig. S2).

Next, to address our hypothesis that knock-down of MnSOD leads to elevated blood pressure, we measured hemodynamics in the MnSOD^{L/L} ROSA-Cre^{+/-} mice by radiotelemetry. In contrast to our original hypothesis, we observed no change in baseline mean arterial pressure (MAP) at any time point after knock-down of MnSOD (Fig. 2). Moreover, the AngII-induced increase in MAP was not significantly

different between MnSOD^{L/L} ROSA-Cre^{-/-} and MnSOD^{L/L} ROSA-Cre^{+/-} (Fig. 2). Taken together, these unexpected results indicate that knock-down of MnSOD in peripheral tissues is not sufficient to alter MAP in the presence or absence of AngII.

3.2. Increased mitochondrial superoxide in the brain sensitizes mice to AngII

Due to the observation that the MnSOD^{L/L} ROSA-Cre^{+/-} mice did not elicit any change in MnSOD protein in the brain, we pursued the development of a mouse possessing MnSOD knock-down targeted to the brain SFO; a blood-brain barrier deficient cardiovascular control nuclei known to be sensitive to circulating AngII [21,22]. To achieve this, we localized cre-recombinase delivery via intracerebroventricular (ICV) injection of adenovirus (AdCre or AdControl) in the parental MnSOD^{L/L} mice (Fig. 3A).

To characterize the knock-down of MnSOD in the SFO, we utilized laser-capture microdissection to specifically isolate this unique and diminutive brain region. Quantitative real-time PCR analysis demonstrated an approximate 60% decrease in MnSOD mRNA in the SFO 14 days after AdCre administration (Fig. 3B). We next examined if the loss of MnSOD confined to a specific cardiovascular control region in the brain would be sufficient to alter peripheral MAP. Interestingly, 14 days after the delivery of AdCre (at which point MnSOD expression is decreased, Fig. 3B), no change in baseline MAP was observed compared to mice ICV-treated with AdControl (Fig. 3C). However, peripheral infusion of AngII elicited a significantly greater response in AdCre-treated animals compared to AdControl, suggesting the loss of MnSOD in the SFO sensitizes mice to AngII hypertension (Fig. 3C). In congruence with this, once the AngII minipumps emptied (~18 days after the start of AngII infusion), MAP in both AdCre and AdControl mice returned to baseline with no significant differences noted (Fig. 3C).

Next, we examined the $O_2^{\bullet-}$ levels in the SFO during the AngII infusion time course. After only 2 days of AngII infusion (point at which the hemodynamics remain significantly different from control throughout the infusion) intracellular $O_2^{\bullet-}$ levels were significantly higher in the AdCre treated mice versus AdControl mice infused with AngII as evidenced by both DHE and MitoSOX oxidation (Fig. 4A and B). Overall, these data correlate with the observed changes in systemic hemodynamics and suggest that decreased MnSOD and amplified mitochondrial $O_2^{\bullet-}$ specifically in the SFO of the brain increases the sensitivity to circulating hypertensive stimuli such as AngII.

3.3. Over-expression of MnSOD in the brain attenuates AngII-mediated hypertension

With the observation that increased mitochondrial $O_2^{\bullet-}$ in the SFO increases the AngII hypertensive response, we wanted to examine the causal role of this ROS in enhancing the response to hypertensive stimuli. To this end, we developed a model of decreased mitochondrial $O_2^{\bullet-}$ by over-expressing MnSOD via adenovirus (AdMnSOD) specifically in the SFO of wild-type mice (Fig. 5A).

Characterization of the SFO from AdMnSOD-injected mice demonstrated an approximate 70-fold increase in MnSOD mRNA levels compared to AdControl (Fig. 5B), thus, confirming adenovirus-mediated gene transfer and enhanced expression of MnSOD. Adenovirus-mediated transgene expression in the SFO was further supported by robust immunostaining of both GFP and MnSOD within the SFO of AdGFP- (AdControl) and AdMnSOD-injected mice, respectively (Fig. 5C).

As previously observed in the other models of MnSOD perturbation, over-expression of MnSOD in the SFO has no impact on baseline MAP (Fig. 5D). In contrast, peripheral infusion of AngII significantly increased MAP in AdControl mice, but failed to elicit the complete hypertensive response in mice over-expressing MnSOD in the SFO

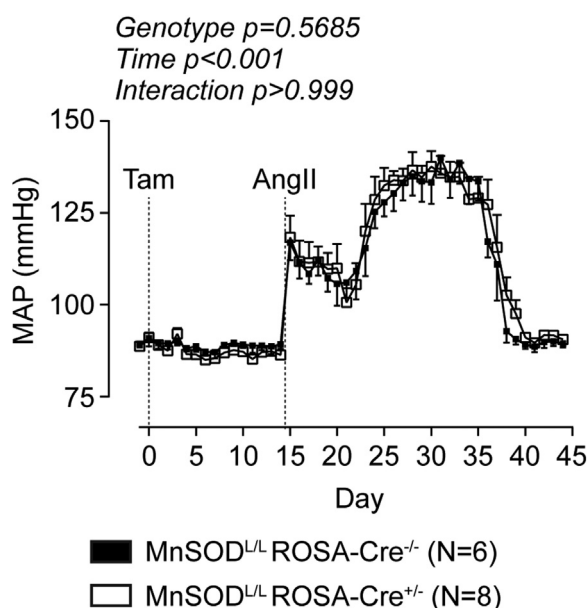


Fig. 2. Peripheral MnSOD knock-down does not affect baseline or AngII-mediated changes in MAP. Mice were treated with tamoxifen (150 mg/kg) daily for 6 days followed by a 14-d incubation period to allow endogenous MnSOD to turnover prior to subcutaneous AngII infusion (400 ng/kg/min). Daily averages of telemetric MAP data acquired over the course of the experiment. Tam indicates final day of tamoxifen administration. 2-way ANOVA followed by Bonferroni correction post hoc test was utilized for statistical analysis.

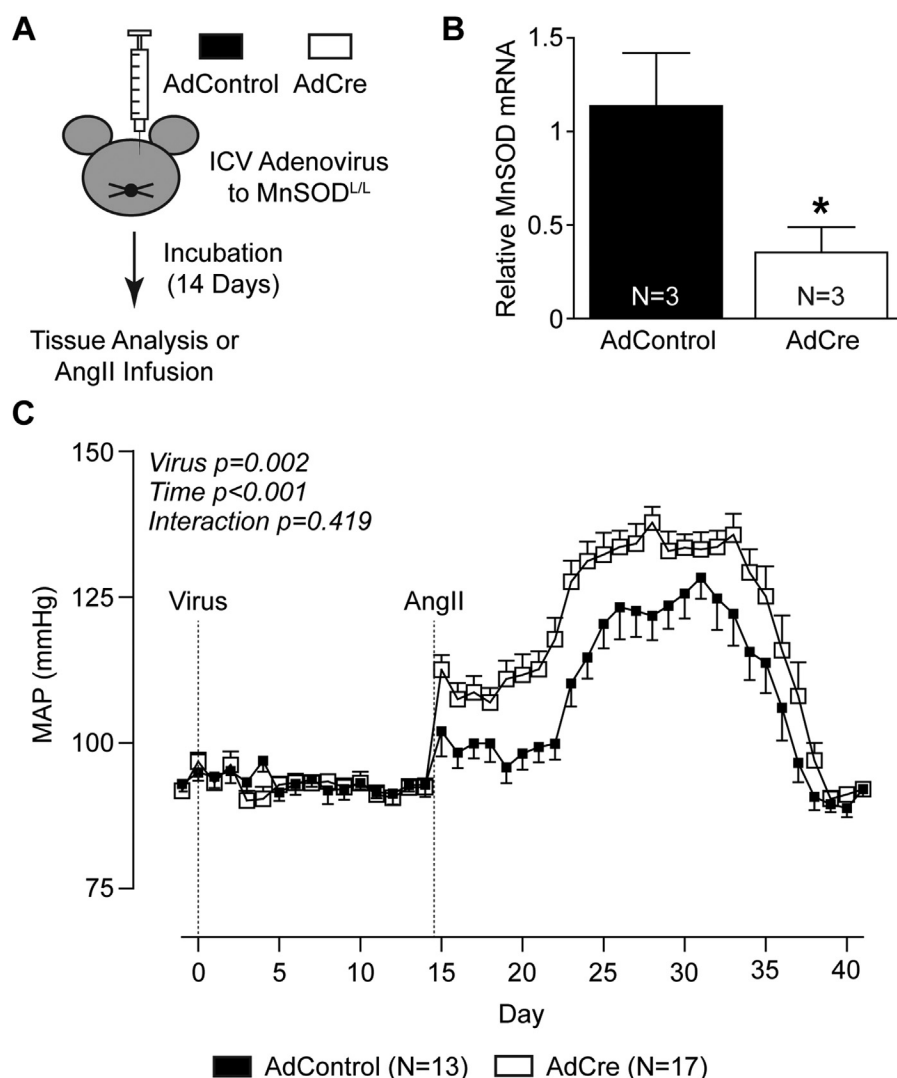


Fig. 3. MnSOD knock-down in the SFO sensitizes mice to chronic AngII-mediated hypertension. Mice were treated with a one-time ICV injection of either AdCre or AdControl virus followed by a 14-d incubation period to allow endogenous MnSOD to turnover prior to tissue harvest. **A.** Schematic of experimental setup and timeline. **B.** Quantitative real-time RT-PCR analysis of MnSOD mRNA from SFO tissue obtained via laser microdissection. * $p < 0.05$ vs. AdControl. **C.** Daily averages of telemetric MAP data acquired over the course of the experiment. 2-way ANOVA followed by Bonferroni correction post hoc test was utilized for statistical analysis.

(Fig. 5D). Additionally, after AngII had dissipated from the mini-pumps, no significant differences in MAP were noted in either animal model (Fig. 5D). Additionally, in response to AngII, $O_2^{\cdot-}$ levels measured by DHE and MitoSOX fluorescence were attenuated in AdMnSOD-treated mice versus AdControl (Fig. 6A and B). In summary, the attenuation of mitochondrial $O_2^{\cdot-}$ in the brain SFO via over-expression of MnSOD desensitizes mice to chronic, peripheral AngII challenge.

4. Discussion

The present study elucidated two major findings. First, knock-down of MnSOD, the mitochondrial-localized $O_2^{\cdot-}$ scavenging enzyme, in peripheral tissues or the brain SFO is not sufficient to alter MAP under basal conditions, and second, perturbations of mitochondrial $O_2^{\cdot-}$ in the SFO, but not peripheral organs, sensitize mice to AngII-dependent hypertension. While increases in mitochondrial $O_2^{\cdot-}$ have been observed in various cell types in response to AngII [2,7–9,23–25], these findings raise the question if certain cell types and/or organs are more dependent upon this mitochondrial-localized ROS than others when perpetuating the AngII signaling cascade.

Previous studies have demonstrated that AngII increases mitochon-

drial $O_2^{\cdot-}$ in neurons and that over-expression of MnSOD is able to reverse AngII-mediated responses in these cells suggesting a causal role for mitochondrial $O_2^{\cdot-}$ in mediating AngII intraneuronal signaling [4–7,9]. Interestingly, we have shown that over-expression of copper zinc superoxide dismutase (CuZnSOD), which is traditionally characterized as being localized to cytoplasm, also reverses the AngII-mediated effects on neurons [18]. While this may suggest a non-mitochondrial source of AngII-derived $O_2^{\cdot-}$, numerous studies have reported that CuZnSOD is expressed in the intramitochondrial membrane space (IMM) [26–29]. We have found that adenovirus-mediated over-expression of CuZnSOD in neurons leads to an accumulation of the enzyme in mitochondria, thus further supporting the idea that this enzyme plays a role in attenuating mitochondrial $O_2^{\cdot-}$ in response to AngII [18]. In addition to our work, others have also observed mitochondrial $O_2^{\cdot-}$ as a primary signaling molecule in AngII-mediated hypertension models. For example, vascular endothelial cells have demonstrated an increase in mitochondrial $O_2^{\cdot-}$ in response to AngII [2,8,23]. This increase in mitochondrial $O_2^{\cdot-}$ led to a dysfunctional vascular tone, disruption of mitochondrial metabolism, and an increase in MAP that could be reversed by the systemic administration of MitoTempol, a mitochondrial-targeted $O_2^{\cdot-}$ scavenger. Overall, these data together suggest a strong role for $O_2^{\cdot-}$ derived in the mitochondria

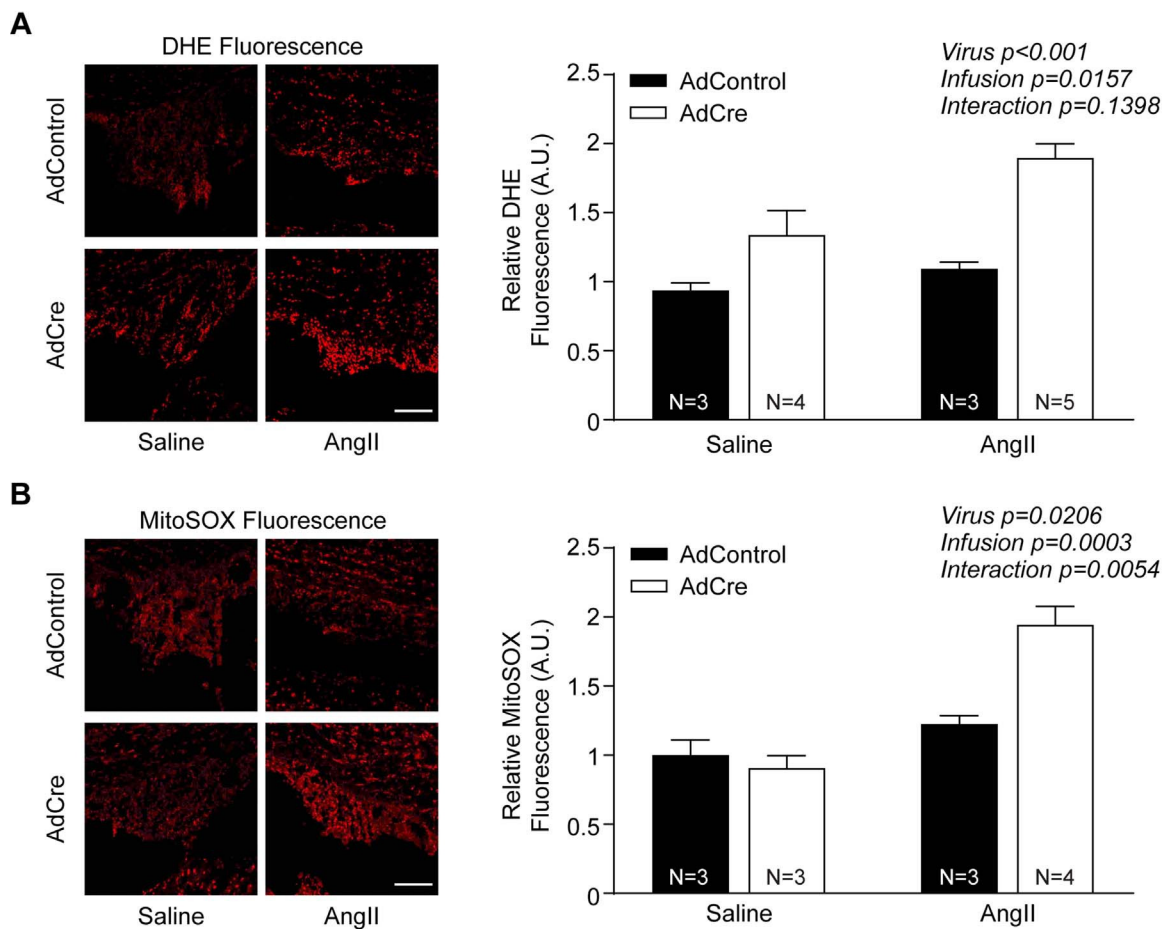


Fig. 4. MnSOD knock-down in the SFO mice elevates mitochondrial $O_2^{\bullet-}$ in response to systemic AngII infusion. Mice were treated with a one-time ICV injection of either AdCre or AdControl virus followed by a 14-d incubation period to allow endogenous MnSOD to turnover prior to subcutaneous AngII infusion (400 ng/kg/min). Tissue was isolated 2 days following AngII infusion. **A.** Left, representative confocal microscopy images showing DHE fluorescence in the brain SFO from an AdControl or AdCre-injected mouse with or without AngII infusion. Right, quantification of DHE oxidation. **B.** Left, representative confocal microscopy images showing MitoSOX fluorescence in the brain SFO from an AdControl or AdCre-injected mouse with or without AngII infusion. Right, quantification of MitoSOX oxidation. 2-way ANOVA followed by Bonferroni correction post hoc test was utilized for statistical analysis. Scale bar=100 μ m.

in driving AngII-mediated hypertension. However, our work presented here suggests that increasing mitochondrial $O_2^{\bullet-}$ in the absence of AngII is not sufficient to increase MAP, which implies multiple mechanisms in addition to mitochondrial redox regulation are most likely at play in regards to AngII-dependent hypertension.

One possible explanation for why increased mitochondrial $O_2^{\bullet-}$ is not sufficient to increase MAP may be due to the directionality of the $O_2^{\bullet-}$ produced in response to AngII. As previously mentioned, we have shown that over-expression of CuZnSOD is able to attenuate mitochondrial derived $O_2^{\bullet-}$ in response to AngII, and thus has a rescuing effect in neurons [18]. This would suggest that the mitochondrial $O_2^{\bullet-}$ that mitigates the effects of AngII may be primarily the $O_2^{\bullet-}$ produced into the IMM space of the mitochondria as opposed to the matrix. Our study presented herein utilized mouse models possessing increased steady-state $O_2^{\bullet-}$ in the mitochondrial matrix due to the knock-down of MnSOD, and thus this spatial difference in ROS production may lead to differential regulatory control of cellular processes. However, this theory is convoluted by the numerous observations that MnSOD over-expression, and thus lowering of mitochondrial matrix $O_2^{\bullet-}$, may also attenuate AngII-mediated signaling [7,8,10]. Another possible explanation may be that in addition to increasing mitochondrial-localized $O_2^{\bullet-}$, AngII recruits redox-regulated proteins necessary for signal transduction in close proximity to the mitochondria. We have shown that Ca^{2+} /calmodulin kinase II (CaMKII), a known redox-regulated enzyme [30], becomes activated in response to AngII and this activation is reversed by the over-expression of MnSOD [7]. Others

have demonstrated that the enzyme protein kinase C (PKC) also contributes to AngII signaling [2], therefore increasing the number of potential redox-sensitive components that may be interacting with AngII-mediated mitochondrial $O_2^{\bullet-}$. Thus, simply increasing mitochondrial $O_2^{\bullet-}$ (via MnSOD knock-down) without altering the activity of these signaling proteins would not reproduce the same effect elicited by AngII stimulation, and may explain why baseline hemodynamics were not affected in any of our mouse models.

Another possible reason increased mitochondrial $O_2^{\bullet-}$ alone did not change baseline blood pressure may be due to the regions in which the MnSOD knock-down was targeted. It has been previously observed that increased mitochondrial $O_2^{\bullet-}$ in the rostral ventrolateral medulla (RVLM), another cardiovascular control region in the brain, increases systemic blood pressure [4–6]. In spontaneous hypertensive rats (SHR), these animals have been shown to possess decreased expression of MnSOD and increased mitochondrial $O_2^{\bullet-}$ specifically in the RVLM of the brain [5]. This increase in mitochondrial $O_2^{\bullet-}$ appeared partially causal to the phenotypic hypertension as over-expression of MnSOD specifically in the RVLM was able to lower systemic mean arterial pressure in these animals [5]. Moreover, administration of the mitochondrial complex I inhibitor rotenone, which further increases mitochondrial $O_2^{\bullet-}$, to the RVLM was able to potentiate the systemic hypertension in these animals [5]. The brain RVLM is a primary integration site in the brain stem regulating sympathetic outflow [31], thus perturbations of mitochondrial $O_2^{\bullet-}$ in the RVLM may affect sympathetic drive, which would have significant impacts on peripheral

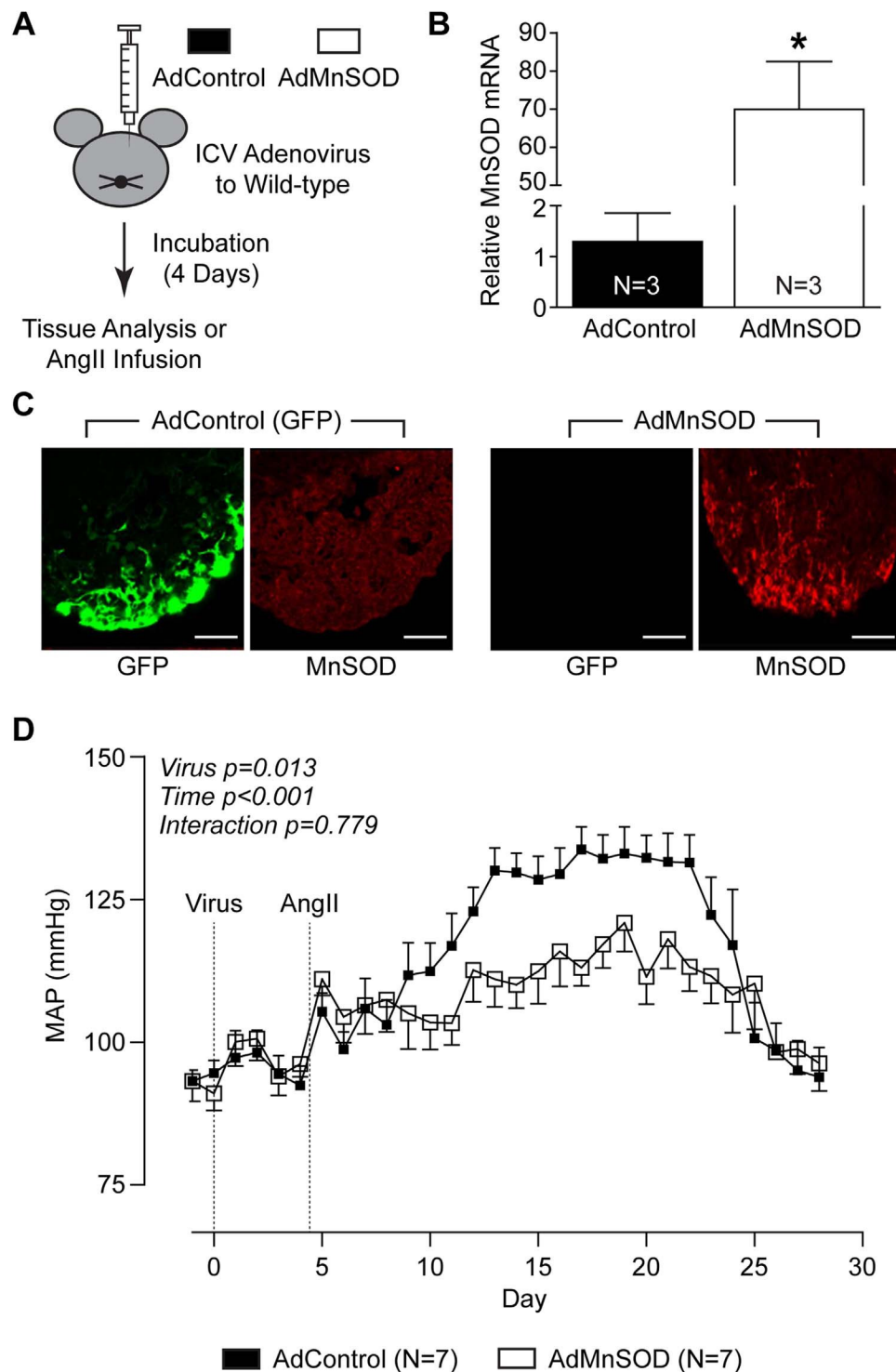


Fig. 5. Over-expression of MnSOD in the SFO decreases peripheral blood pressure in response to chronic AngII-mediated infusion. Mice were treated with a one-time ICV injection of either AdMnSOD or AdControl virus followed by a 4-d incubation period to allow exogenous MnSOD to be expressed prior to tissue harvest. **A.** Schematic of experimental setup and timeline. **B.** Quantitative real-time RT-PCR analysis of MnSOD mRNA from SFO tissue obtained via laser microdissection. * $p < 0.05$ vs. AdControl. **C.** Representative confocal microscopy images of GFP and MnSOD protein expression in the brain SFO from an AdControl (GFP) or AdMnSOD-injected mouse. **D.** Daily averages of telemetric MAP data acquired over the course of the experiment. 2-way ANOVA followed by Bonferroni correction post hoc test was utilized for statistical analysis. Scale bar = 100 μ m.

vascular tone and blood pressure regulation. In the work presented herein, knock-down of MnSOD in the brain was primarily confined to the SFO. The SFO does not directly project to the RVLM [32], and as such may explain why increases in mitochondrial $O_2^{\bullet-}$ in the SFO in the absence of hypertensive stimuli may not elicit the same hemodynamic phenotype.

While we discovered that increased mitochondrial $O_2^{\bullet-}$ alone did

not affect baseline blood pressure in our animals, we did observe that increases in central mitochondrial $O_2^{\bullet-}$, particularly in the brain SFO, did in fact sensitize the mice to AngII-mediated hypertension. This may be due to inherent cell type-specific differences in AngII-signaling. For example, we have recently observed that a source of mitochondrial $O_2^{\bullet-}$ in AngII-stimulated neurons is NADPH oxidase 4 (NOX4) [9]. We observed that NOX4 is partially localized to the mitochondria, and that

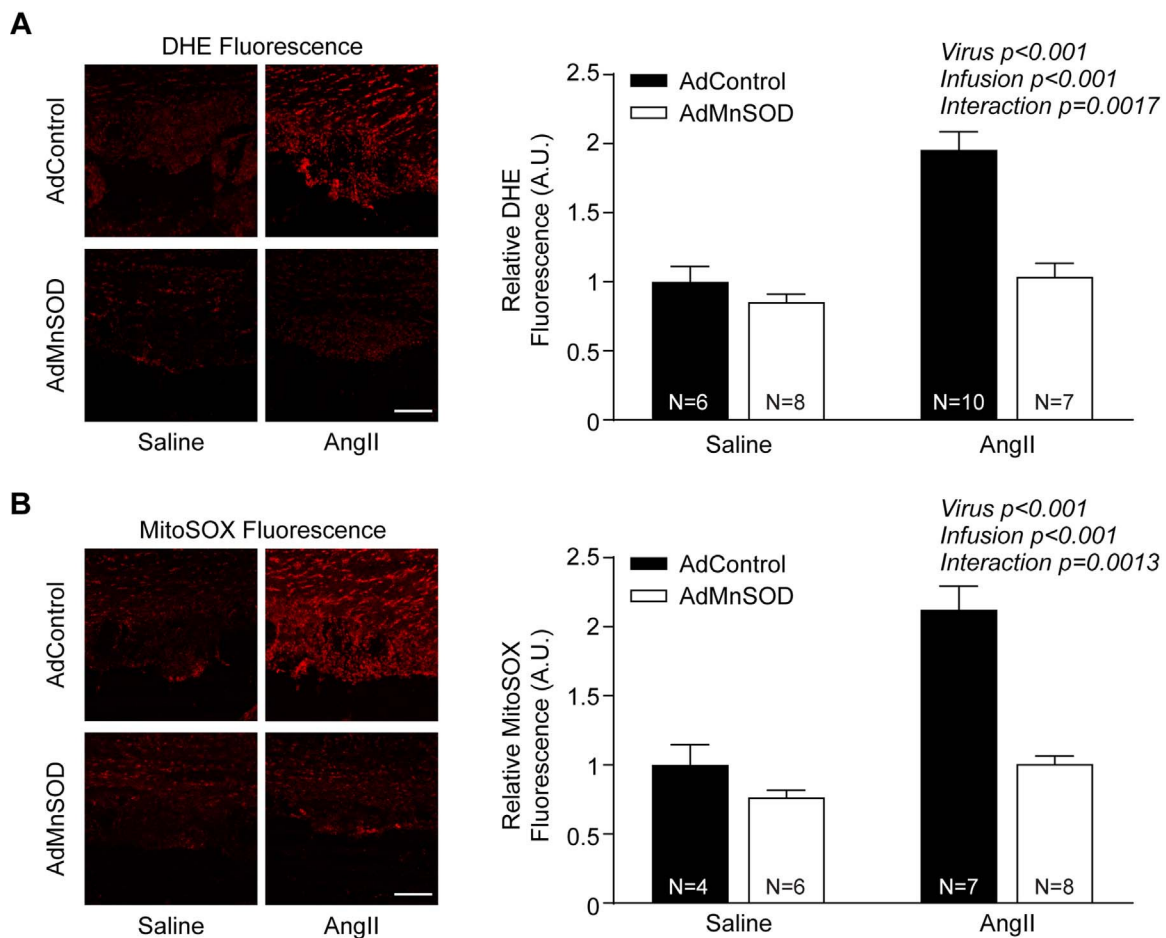


Fig. 6. MnSOD over-expression in the SFO mice decreases mitochondrial $O_2^{\bullet-}$ in response to systemic AngII infusion. Mice were treated with a one-time ICV injection of either AdMnSOD or AdControl virus followed by a 4-d incubation period to allow exogenous MnSOD to be expressed prior subcutaneous AngII infusion (400 ng/kg/min). Tissue was isolated 14 days following AngII infusion. **A.** Left, representative confocal microscopy images showing DHE fluorescence in the brain SFO from an AdControl or AdMnSOD-injected mouse with or without AngII infusion. Right, quantification of DHE oxidation. **B.** Left, representative confocal microscopy images showing MitoSOX fluorescence in the brain SFO from an AdControl or AdMnSOD-injected mouse with or without AngII infusion. Right, quantification of MitoSOX oxidation. 2-way ANOVA followed by Bonferroni correction post hoc test was utilized for statistical analysis. Scale bar=100 μ m.

siRNA down-regulation of NOX4 attenuates AngII-mediated mitochondrial $O_2^{\bullet-}$ production in cultured neurons [9]. In contrast, in endothelial cells NOX4 appears not to be localized to mitochondria, and moreover, NOX2 was demonstrated to be a primary contributor to the generation of mitochondrial $O_2^{\bullet-}$ [23]. These cell type specific nuances may partially explain why the loss of MnSOD in the central nervous system and not peripheral tissues sensitizes mice to AngII. Another potential explanation for the central versus peripheral discrepancy may be due to cellular function. We have previously shown that AngII-mediated mitochondrial $O_2^{\bullet-}$ inhibits neuronal potassium current [7], and thus increases neuronal firing. It may be possible that the loss of MnSOD and ensuing increase in mitochondrial $O_2^{\bullet-}$ has a similar effect on neurons located in the SFO, and that the addition of AngII further exacerbates this effect leading to potentiated neuronal firing. Due to SFO neurons playing a major role in the regulation of cardiovascular function [21,32,33], this may explain why systemic MAP increases are exaggerated in response to AngII only in the SFO knock-down of MnSOD. In contrast, knock-down of MnSOD and increases in mitochondrial $O_2^{\bullet-}$ in peripheral tissues may not influence central neuronal firing, and as such are not sufficient to sensitize to AngII-induced systemic hemodynamic changes.

5. Conclusions

Hypertension is a multifactorial disease that is associated with

increases in redox signaling. However, the use of antioxidants in the amelioration of the disease has proved unsuccessful to date [34]. One possible explanation for this failure is that the antioxidants currently utilized do not target the appropriate cell type, subcellular location, or specific ROS produced that perpetuates the hypertension. We and others have shown that mitochondrial $O_2^{\bullet-}$ is a specific ROS necessary in the pathogenesis of hypertension [2,8–10,18,23]. While whole body administration of mitochondria-targeted $O_2^{\bullet-}$ scavengers or over-expression of MnSOD has proven beneficial in the attenuation of AngII-mediated experimental hypertension [8], these findings have not elucidated the specific tissues in which these mitochondrial-localized antioxidants are eliciting their effects. Our work here demonstrates that increased mitochondrial $O_2^{\bullet-}$ in peripheral tissues or the brain is not sufficient to alter systemic hemodynamics, but increases in mitochondrial $O_2^{\bullet-}$ in the brain SFO potentiate AngII-mediated hypertension. These findings advance the understanding of the specifics behind ROS in elevated blood pressure, and have elucidated a primary organ (*i.e.* the brain SFO) and subcellular (*i.e.* mitochondria) target, as well as a specific ROS (*i.e.* $O_2^{\bullet-}$) for therapeutic intervention in the treatment of hypertension associated with elevated levels of AngII.

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Conflict of interest/disclosures

None.

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Appendix A. Supplementary Information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.redox.2016.11.011>.

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